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Peptides Controlling Stiffness of Connective Tissue in Sea Cucumbers

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Abstract. We present the first evidence of a system of four bioactive peptides that affect the stiffness of sea cucumber dermis. The body wall dermis of sea cucumbers consists of catch connective tissue that is characterized by quick and drastic stiffness changes under nervous control. The peptides were isolated from the body wall, their amino acid sequences determined, and identical peptides synthesized. Two peptides, which we named holokinins, are homologous with bradykinin. We tested the effect of the peptides on the mechanical properties of sea cucumber dermis. Both of the holokinins softened the dermis, and a pentapeptide that we designated as NGIWYamide stiffened it. Both effects were reversibly suppressed by anesthesia with menthol. We called the fourth peptide stichopin; it had no direct effect on the stiffness of the dermis but suppressed action of the neurotransmitter acetylcholine reversibly. The results suggest that the peptides are neuropeptides and are part of a sophisticated system of neurotransmitters and neuromodulators that controls the connective tissue stiffness of sea cucumber dermis.

Introduction

One key character of echinoderms is their unique catch connective tissue (CCT, Motokawa, 1984a; mutable connective tissue, Wilkie, 1984). This connective tissue can change its mechanical properties dramatically in response to various stimuli. The response, which can be stiffening or softening, is under nervous control.

The body wall of sea cucumbers consists of a thin epidermis, an extensive dermis, and inner muscles composed of a sheet of ring muscles and five bands of longitudinal muscles. In most species the dermis makes up by far the biggest part of the body wall. The dermis consists of pure CCT and can change its properties to the extent that the animal can be very stiff to the touch at one moment and practically melt a few seconds later (Motokawa, 1988); in extreme cases, drastic softening leads to autotomy. A number of studies reported different stimuli that lead to stiffness changes (Motokawa, 1981, 1982c, 1984b, 1984c, 1987; Hayashi and Motokawa, 1986). Among them are acetylcholine (ACh) and cholinergic agonists/antagonists. Other common vertebrate neurotransmitters did not have any effect. The holothurian nervous system is very poorly known, and—like all echinoderm nervous systems—it seems to lack synapses. The site of action of ACh is unknown, as is the mechanism of neural transmission in echinoderms.

Many studies of vertebrates have shown the importance of neuropeptides in neural transmission. In echinoderms, only a few peptides have been isolated and classified as neuropeptides. The sea star *Asterias rubens* has two peptides: S1 (Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-NH_2) and S2 (Ser-Gly-Pro-Tyr-Ser-Phe-Asn-Ser-Gly-Leu-Thr-Phe-NH_2) (Elphick *et al.*, 1991). The second of these (S2) has a relaxing effect on the cardiac stomach of *A. rubens* (Elphick *et al.*, 1995). The sea cucumber *Holothuria glaberrima* has two peptides: SGYSVLYamide and GFSKLYFamide (Diaz-Miranda *et al.*, 1992). The latter induces relaxation of the intestinal muscles and the longitudinal body wall muscles of *H. glaberrima.* No other bioactive peptides are known at present, and no study has investigated the effects of peptides on connective tissue stiffness. However, there are pioneering studies that sug-

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gest the presence of low molecular weight factors controlling the stiffness of echinoderm connective tissues (Smith and Greenberg, 1973; Motokawa, 1981, 1982a, 1982c, 1984b). These studies reported a stiffening factor and a softening factor in coelomic fluids and body wall of echinoderms but did not analyze its chemical structure.

On the other hand, Iwakoshi *et al.* (1995) isolated a number of peptidic bioactive substances from the body wall of the sea cucumber *Stichopus japonicus* and determined the structures of four peptides. They did not, however, test the influence of those peptides on the connective tissue of sea cucumber. We recently made further attempts to isolate bioactive peptides from the body wall of sea cucumber and found 14 peptides, including the four obtained in the previous study. A preliminary account of the identification process is given in Ohtani *et al.* (1998). These peptides have been tested on the connective tissue of the sea cucumber. To date, we have found that four of them (termed holokinin 1 and 2, NGIWYamide, and stichopin) have considerable effects on the stiffness of the body wall connective tissue; these effects are reported here.

Materials and Methods

Specimens of the sea cucumber *Stichopus japonicus* were collected in the Seto Inland Sea near Hiroshima and in the Japan Sea near the Noto peninsula. Specimens of *Holothuria leucospilota* were collected in Okinawa. The animals were kept in aquaria with circulating seawater at 18°C and 25°C, respectively.

Isolation and structure determination of peptides

Body walls, including longitudinal muscles, from a 5-kg sample of *S. japonicus* were cut into small pieces, frozen in liquid nitrogen, and pulverized. The pulverized material was boiled for 10 min in 10 volumes of water, then homogenized with a Waring blender and a Polytron. The homogenate was centrifuged (13,000 × *g*, 40 min, 4°C). The supernatant was concentrated to 200 ml with an evaporator. To the concentrated supernatant, 20 ml of 1 *N* HCl was added and the precipitate was centrifuged off. Next, the supernatant was forced through a series of five C18 cartridges (Varian Mega Bond-Elut). The retained material was eluted with 10% and then with 60% methanol. The 60% methanol eluate was subjected to HPLC purification. The four peptides used in the present experiments were purified through five or six HPLC steps with two kinds of C18 reversed-phase columns (Asahikasei ODP-50 and Tosoh ODS-80TM) and a cation-exchange column (Tosoh SP-5PW). After each purification step, the bioactivity of the obtained fractions was assayed on longitudinal muscle and intestine of *S. japonicus*. Details concerning the purification of the four active peptides found in this study are given in the Results.

Bioassay

A longitudinal muscle (10-mm length) was dissected out, tied with cotton thread at each end, and mounted in a chamber (2.5 ml) filled with artificial seawater (ASW). The ASW used in the bioassay was of the following composition: 445 m*M* NaCl, 10 m*M* KCl, 10 m*M* $CaCl_2$, 55 m*M* $NaCl_2$, and 10 m*M* Tris-HCl (pH 7.6). Twitch contraction of the muscle was elicited by stimulating it with an electrical pulse (20 V, 3 ms) at 10-min intervals. The stimulation was delivered in air after removing the ASW in the chamber. After each recording of twitch contraction, the chamber was again filled with ASW. Peptide samples were dissolved in ASW and applied to the muscle 8 min before stimulation. The intestine (75-mm length) was mounted in another chamber. Peptide samples were dissolved in 0.1 ml ASW and applied to the intestine by injection into the aerated ASW.

Mechanical tests

For oscillatory strain tests, tissue samples (1 mm × 1 mm × 5 mm) were cut from the interambulacral white dermis with a razor blade. The long axis of the sample corresponded to the long axis of the animal. The samples were glued to a holder in a trough filled with ASW or experimental solutions. The samples were connected to a force gauge (LVS-20GA, Kyowa), whose position oscillated between 0 and 800 μm at a rate of 0.3 Hz. The samples were thus periodically stretched and relaxed along their long axis. The oscillator was adjusted so that the sample was totally relaxed in one half cycle but was never compressed. The samples developed a counterforce that oscillated with the same period as the strain. The counterforce was recorded with a microcomputer at a rate of 7 or 10 Hz. Chemicals were diluted in ASW and added to the trough.

For creep tests, samples similar to those used for oscillatory strain tests were glued to a holder in a trough. Loads between 0.09 g and 5 g were applied *via* a lever system to ensure constant elongation of the sample. The elongation of the sample was measured with a laser displacement sensor (3Z4M-S01, Omron) and recorded with a microcomputer. Chemicals were diluted in ASW to a concentration of 10^{-6} *M* (for most experiments) or 10^{-5} *M* (in a few cases) and added to the trough. Relative viscosity was calculated as the reciprocal value of strain rate normalized by the same value at application of test solutions.

The samples of body wall tissue used in the mechanical tests were taken from at least 5 animals for each neuropeptide. We could not find statistically significant differences in responses between animals or between samples from the same animal.

Results

Purification and bioassay of peptides

At the first step of HPLC purification, the column (reversed-phase, ODP-50) was eluted with a 120-min linear gradient of 0%–60% acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) at pH 2.2. The results of bioassay experiments showed that most fractions obtained between 10% and 34% ACN had effects on the contractility of both the longitudinal muscle and the intestine. Therefore, we divided the active fractions into six groups. Two of the four peptides used in the present study were purified from group 1, which was obtained between 10% and 14% ACN. The other two were purified from group 2, which was obtained between 14% and 18% ACN.

Group 1 was applied to a cation-exchange column, and the column was eluted with a 70-min linear gradient of 0–0.7 *M* NaCl in 10 m*M* phosphate buffer at pH 7.1. An active peak (P1), which was found to have an inhibitory effect on the twitch contraction of the radial muscle but no effect on spontaneous contractions of the intestine, eluted at about 0.15 *M* NaCl. Another active peak (P2), which was found to have a contractile effect on both muscle tissues, eluted at about 0.25 *M* NaCl. P1 was applied to another kind of reversed-phase column (ODS-80TM), and an active peak eluted at about 18% ACN. The peak was applied to the same column, and the column was eluted isocratically with 16% ACN. To purify the active substance of the peak, isocratic elution was repeated under the same conditions. P2 was also applied to the ODS-80TM column. The active peak eluted at about 20% ACN. Next, the peak was again applied to the cation-exchange column. Although the conditions of elution were the same as in the second step, the active peak eluted at about 0.1 *M* NaCl. To purify the active substance of P2, isocratic elution was performed twice with the ODS-80TM column at 19% ACN.

Group 2 was also applied to the cation-exchange column, and the column was eluted under the same conditions as for group 1. An active peak, which was found to have an inhibitory effect on twitch contraction of the radial muscle and a contractile effect on the intestine, eluted at about 0.3 *M* NaCl. The peak was then applied onto an ODS-80TN column. Two overlapping active peaks (P3 and P4) eluted at about 22% ACN. Both peaks showed similar activities. Next, P3 was applied to the same column, and the column was eluted isocratically with 21% ACN. The isocratic elution was again performed and the P3 substance was purified. To purify the P4 substance, similar isocratic elution with 21.5% ACN was performed twice.

The purified peptidic substances were subjected to amino acid sequence analysis and fast atom bombardment mass spectrometry (FAB-MS). The results of the analyses of the substances and their structures predicted from the results are shown in Figure 1. P1 was suggested to be a heptadecapeptide having a disulfide bond. The peptide was named stichopin. P2 was suggested to be a pentapeptide with an amidated C-terminus. The peptide was named NGIWYamide according to its structure. P4 was suggested to be a bradykinin-related heptapeptide. Its predicted structure is the same as holokinin 1 found in a previous study (Iwakoshi *et al.*, 1995). P3 was suggested to be Met(O)^5-holokinin 1; that is, the peptide has an oxidized methionine at the fifth position of holokinin 1. This peptide has also been found in the previous study and was termed holokinin 2. It cannot be ruled out that

Holokinin 1

Sequence Pro-Leu-Gly-Tyr-Met-Phe-Arg

FAB-MS 883.5 $(M + H)^+$

Structure Pro-Leu-Gly-Tyr-Met-Phe-Arg

Holokinin 2

Sequence Pro-Leu-Gly-Tyr-Met-Phe-Arg

FAB-MS 899.4 $(M + H)^+$

Structure Asn-Gly-Ile-Trp-Tyr-NH_2

NGIWYamide

Sequence Asn-Gly-Ile-Trp-Tyr

FAB-MS 651.2 $(M + H)^+$

Structure Asn-Gly-Ile-Trp-Tyr-NH_2

Stichopin

Sequence Asp-Arg-Gln-Gly-Trp-Pro-Ala-Xaa-Tyr-Asp-Ser-Lys-Gly-Asn-Tyr-Lys

FAB-MS 1989.7 $(M + H)^+$

Structure

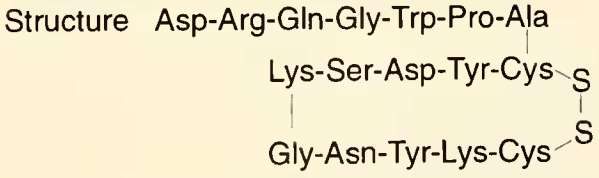

Figure 1. Results of amino acid sequence analysis and fast atom bombardment mass spectrometry (FAB-MS) of the purified peptides, and the structures predicted from the results.

the oxidization occurred during the purification of holokinin 1, though holokinin 2 (oxidized holokinin 1) had an almost identical effect on the muscle tissues and connective tissue.

Peptides with the predicted structures were synthesized. The behavior of each synthetic peptide on reversed-phase and cation-exchange HPLC was confirmed to be identical with that of the corresponding native peptide. Further, the effects of each synthetic peptide on the radial muscle and the intestine were also confirmed to be identical with those of the corresponding native peptides.

Figure 1 shows the results of amino acid sequence and FAB-MS analysis as well as the predicted structure of the four peptides isolated from the body wall of *Stichopus japonicus*. We used synthetic peptides to test whether they have an influence on the stiffness of sea cucumber connective tissue either by themselves, or by influencing the effect of ACh. Dermis samples of *S. japonicus* were in general softer than those of *H. leucospilota*. However, this did not influence the reactivity of the samples, and we did not observe any significant differences among the species.

Mechanical tests

The holokinins had a softening effect, whereby the effect of a concentration of $10^{-5}M$ did not differ significantly from that of $10^{-6}M$. The effect could be observed within 30 s after application of holokinins in both kinds of test. In oscillating tests (Figs. 2, 4) the samples developed a maximal counterforce that was more or less constant without stimulation. When holokinins were applied the counterforce dropped sharply, indicating that the tissue softened. In creep tests (Figs. 3, 5), creep velocity increased after application of holokinins, shown in our graphs as a decrease of relative viscosity compared to viscosity at time of application.

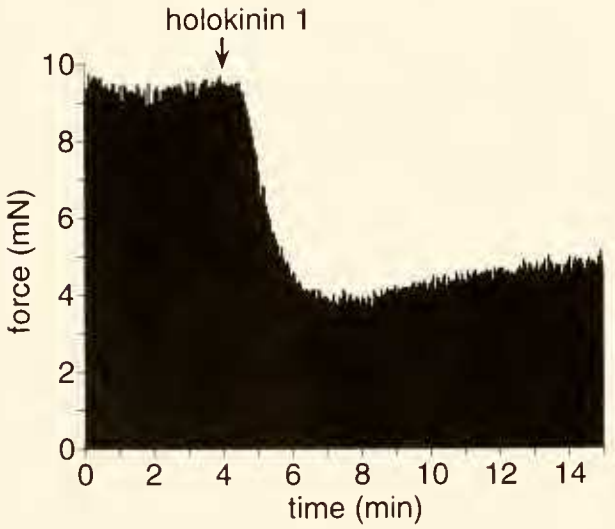

Figure 2. Effect of holokinin 1 ($10^{-6}M$) on stiffness of the dermis of *Holothuria leucospilota* in the oscillatory strain test. The maximal counterforce decreased rapidly after the holokinin 1 was applied. In this graph and in Figures 4 and 6, the time axis is compressed so that it does not resolve each oscillating cycle of the counterforce. Therefore the space between no force and maximal force is blackened entirely in the graphs.

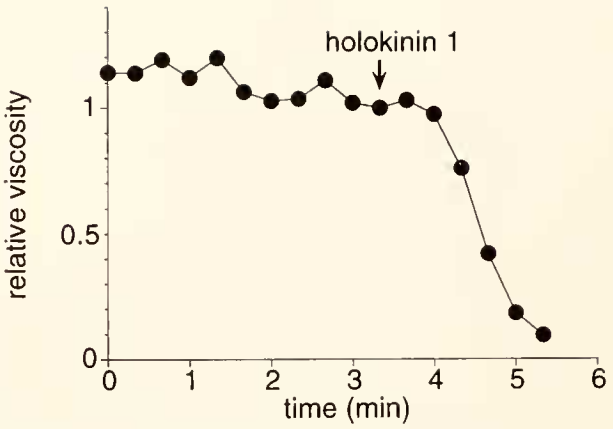

Figure 3. Effect of holokinin 1 ($10^{-6}M$) on the relative viscosity of a dermis sample of *Stichopus japonicus* in the creep test. Relative viscosity was calculated as the reciprocal value of strain rate normalized by the same value at the time when holokinin 1 was applied.

Holokinin 1 at concentrations of $10^{-6}M$ and $10^{-5}M$ softened 19 out of 41 samples. The effect was seen with dermis strips of *H. leucospilota* (Fig. 2) and *S. japonicus* (Fig. 3). Relative viscosity dropped to maximally about 10% in creep tests. Most of the samples stayed soft after holokinin 1 was washed out with ASW. In some cases a slight stiffening (recovery) was observed, but never to the level before application of holokinin 1. In oscillation tests, maximal counterforce dropped maximally to 40% with the application of holokinin 1, and no recovery was observed after washing out with ASW.

Holokinin 2 was slightly less effective, softening 7 out of 24 samples of *H. leucospilota* (Fig. 4) and *S. japonicus* (Fig. 5). In creep tests, relative viscosity dropped to maximally 10% without recovery, and in oscillation tests, maximal counterforce was reduced to 23% after application of holokinin 2.

When we treated samples for 10 min in ASW saturated with menthol, the softening reaction of both holokinins was suppressed, but recovered at least partly after extensive washing in ASW.

The neuropeptide bradykinin, common in vertebrates and wasps (Elliott *et al.*, 1960; Nakajima, 1988) was also tested, because it is partly homologous in sequence to the holokinins. However, it had no effect on the stiffness of holothurian dermis, nor did it suppress or enhance the effect of ACh.

NGIWYamide had the opposite effect from that of the

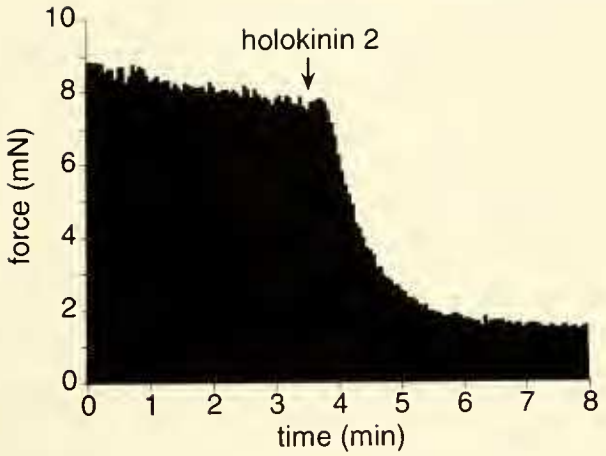

Figure 4. Effect of holokinin 2 (10^{-6} *M*) on the stiffness of the dermis of *Holothuria leucospilota* in the oscillatory strain test.

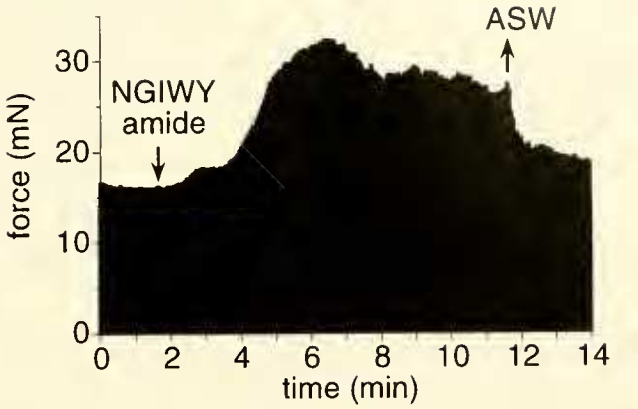

Figure 6. NGtWYamide (10^{-6} *M*) stiffened the dermis of *Holothuria leucospilota* as seen here by an increase in counterforce in the oscillatory strain test. Washing with artificial seawater (ASW) lowered the counterforce to nearly the initial value.

holokinins: it stiffened the dermis in 52 out of 120 samples of both sea cucumber species. Compared to the holokinins, NGIWYamide had a longer reaction time (the reaction occurring within 100 s of application), the reaction was often slower, and the extent of stiffening was very variable. In oscillation tests the maximal counterforce increased, showing a stiffening of the sample (Fig. 6) to maximal 194%. Recovery (*i.e.*, softening of the sample) occurred frequently after washing with ASW. In creep tests (Fig. 7) relative viscosity increased to maximal 220% of the initial value, without recovery after washing with ASW.

As in holokinins, anesthesia for 10 min in ASW saturated with menthol blocked the effect of NGtWYamide reversibly.

Stichopin by itself had no effect on dermal stiffness during our tests. However, when samples were treated with stichopin at 10^{-5} or 10^{-6} *M* for 30 min, the effect of ACh was blocked in creep tests and in forced vibration tests of both sea cucumber species (Fig. 8). In controls, ACh had a stiffening effect or a biphasic effect—first stiffening, then softening. Altogether, stichopin completely blocked the effect of ACh in 27 out of 36 cases, and reduced the effect in the remaining 9 cases. When samples were washed with ASW for at least 1 h, the reactivity of ACh recovered to some extent but never regained the levels seen before treatment with stichopin.

Discussion

The amino acid sequences of holokinins are homologous with those of bradykinins in vertebrates and wasps (Elliott *et al.*, 1960; Nakajima, 1988). Vertebrate bradykinins have a wide range of effects, mainly on the central nervous system and on smooth muscles. Bradykinins from

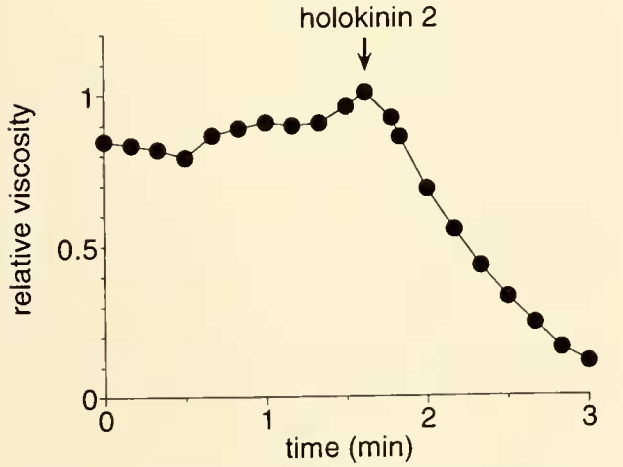

Figure 5. Creep test showing the softening effect of holokinin 2 (10^{-6} *M*) on the stiffness of the dermis of *Stichopus japonicus*.

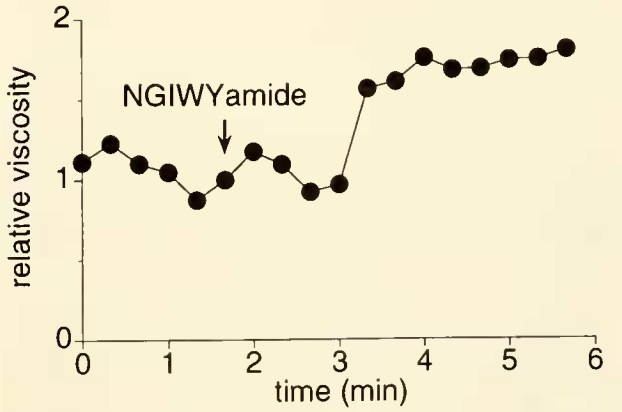

Figure 7. Creep test showing the stiffening effect of NGtWYamide (10^{-5} *M*) on the dermis of *Stichopus japonicus*. Relative viscosity increased *ca.* 1.5-fold.

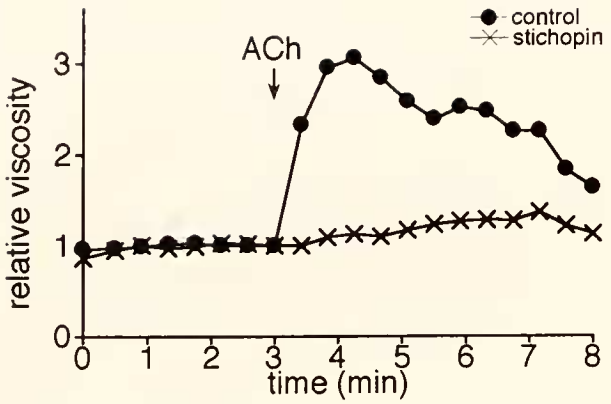

Figure 8. Influence of stichopin on the effect of acetylcholine (ACh) on relative viscosity in the creep test with a sample of *Holothuria leucospilota*. Stichopin was applied at the start of the experiment. At 3 min, 10^{-5} *M* ACh was applied but had no effect (crosses). After washing the sample for 1 h in artificial seawater (ASW), it was tested again as control (circles). Now ACh had a clear stiffening effect, indicating that the suppressive influence of stichopin had disappeared.

wasp venoms show some variation, but the basic bradykinin sequence is well preserved (Nakajima, 1988).

The sequence homology between the holokinins and bradykinin points to the possibility of a common ancestral molecule, although the softening reaction of the sea cucumber connective tissue is unique. Moreover, bradykinin itself did not affect the stiffness of holothurian dermis, so functionally the two peptides are different. The nervous system of sea cucumbers is almost unknown, and speculations about its evolution must wait until more details are available.

Effects

The effect of the peptides was not constant throughout our tests. Dermis samples were previously reported to have different initial physiological states that result in variable responses to the same stimuli (Motokawa, 1984b). These initial states most likely cause variability of responses to peptides.

To test the responses of the dermis, we used two methods that differed in the way the force was applied and in the speed at which the samples elongated. The oscillation test employed repeated stretch-relax cycles and the elongation speed was typically about 500 μm/s. The creep test applied a constant load and the speeds ranged between 0.1 and 3 μm/s. This difference of two orders of magnitude makes it likely that the two tests measure stresses of different parts of the complex molecular network of the connective tissue. The effect of the agents tested did not differ between the test methods, indicating that the peptides affect basic properties of the material that influence tissue mechanics.

Holokinins had the most prominent effect among the peptides tested here and are the first fully characterized softening agent for sea cucumber dermis. Until now, ACh was the only neurotransmitter known to affect dermal stiffness, and its effect is biphasic: that is, it first stiffens and then softens the dermis. As the only identified softening agents, the holokinins are unique. Their effect was easily suppressed by menthol, indicating that they can be classified as neuropeptides.

NGIWYamide is a short peptide and worked antagonistically to the holokinins—it stiffened the dermis. Anesthesia suppressed the effect of NGIWYamide, classifying it as a neuropeptide.

Stichopin lacked an effect when used alone, but suppressed the reaction of sea cucumber dermis to ACh. This suggests that NGIWYamide is also a neuropeptide, modulating the action of ACh.

Studies on factors controlling the stiffness of sea cucumber dermis were pioneered by Smith and Greenberg (1973), who found an evisceration factor with a molecular weight between 131 and 181 in the sea cucumber *Thyone briareus*. Among its other effects, this factor softened the connective tissue of the introvert during evisceration. Later, Motokawa published a series of papers about a softening and a stiffening factor in the coelomic fluid of the sea cucumber *Stichopus chloronotus* (Motokawa, 1981, 1982a, 1982c) and a stiffening factor in the sea cucumber *S. japonicus* (Motokawa, 1984b). The softening factor is heat stable, of low molecular size, and methanol insoluble. The stiffening factor is also heat stable and of low molecular size, but it is methanol soluble. We cannot decide whether the factors found in those studies are identical with the neuropeptides described here, but heat stability and low molecular weight (Iwakoshi *et al.*, 1995) are common characteristics of our neuropeptides. Therefore it seems possible that the two groups are similar or even identical.

Catch connective tissue is unique to echinoderms and characterized by its ability to change stiffness quickly under nervous control without the involvement of muscle cells. The connective tissue of sea cucumber dermis lacks muscle cells entirely, so the observed effects can be attributed to changes of the connective tissue alone. Our data show that four peptides (holokinins, NGIWYamide, and stichopin) compose a system of neuropeptides that can soften and stiffen the dermis and modulate the action of the neurotransmitter ACh. Such a system has not been reported in echinoderms.

It would be interesting to know which cells synthesize and secrete the neuropeptides. Localization of the peptides would probably require specifically tailored antibodies and was far beyond the scope of the present study. Our samples consisted of the white connective tissue of the dermis. Former studies (Motokawa, 1982b; Matsuno and Motokawa, 1992) have shown that this tissue consists of extracellular matrix interspersed with minute processes

of granule-containing cells of various types that resemble vertebrate neurosecretory cells. The peptides in our experiments most likely affected these cells. In a similar fashion, cholinergic drugs evoke stiffness changes in samples of connective tissue (Motokawa, 1987), probably by acting on the same cells. The effect of anesthetics on the action of both peptides and cholinergic drugs (Motokawa, 1987) supports the idea that the granule-containing cells are functionally linked to neural elements. It seems likely that the neuropeptides are synthesized and secreted by these cells in order to closely control the stiffness of sea cucumber dermis.

Other echinoderms

Control of connective tissue stiffness by a peptide or peptide-containing factor has been reported only for the starfish *Pycnopodia helianthoides* (Mladenov *et al.*, 1989). The body fluids of this species contain an autotomy-promoting factor that is most likely a peptide or has a peptide component with a molecular weight of about 1200 daltons. Because of its ability to evoke softening of connective tissue during autotomy, this factor might be similar to the peptides described in our study, although its amino acid composition is unknown.

The other neuropeptides found in echinoderms evoke stomach eversion in starfish (Elphick *et al.*, 1995) or muscle relaxation in sea cucumbers (Diaz-Miranda and Garcia-Arraras, 1995). The latter authors proposed that both the neuropeptides of starfish and the neuropeptide they found in sea cucumbers belong to a family of neuropeptides with a common periodic sequence that includes serine, leucine, and phenylalanine (Diaz-Miranda *et al.*, 1992). The peptides of our study do not fit this amino acid pattern, and we thus conclude that ours are novel neuropeptides different from any known in echinoderms.

Acknowledgments

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